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Regulatory DNA sequences of the gene for the human catalytic telomerase subunit, and their diagnostic and therapeutic use

Structure and function of the chromosome ends

The genetic material of eukaryotic cells is distributed on linear chromosomes. The ends of hereditary units are termed telomeres, derived from the Greek words *telos* (end) and *meros* (part, segment). Most telomeres consist of repeats of short sequences which are mainly composed of thymine and guanine (Zakian, 1995). In all the vertebrates which have so far been investigated, the telomeres consist of the sequence TTAGGG (Meyne *et al.*, 1989).

The telomeres have a variety of important functions. They prevent the fusion of chromosomes (McClintock, 1941) and thus the formation of dicentric hereditary units. Such chromosomes having two centromeres can lead to the development of cancer due to loss of heterozygosis or duplication, or loss of genes.

In addition, telomeres serve the purpose of distinguishing intact hereditary units from damaged hereditary units. Thus, yeast cells ceased their cell division when they contained a chromosome without a telomere (Sandell and Zakian, 1993).

Telomeres fulfil another important task in association with the replication of eukaryotic cell DNA. In contrast to the circular genomes of prokaryotes, the linear chromosomes of eukaryotes cannot be completely replicated by the DNA polymerase complex. RNA primers are required to initiate DNA replication. After elimination of the RNA primers, extension of the Okazaki fragments and subsequent ligation, the newly synthesized DNA strand lacks the 5' end since the RNA primer cannot be replaced by DNA at that point. Without special protective mechanisms, the chromosomes would therefore shrink with each cell division ("end-replication problem"; Harley *et al.*, 1990). The non-coding telomere sequences presumably constitute a buffer zone for preventing the loss of genes (Sandell and Zakian, 1993).

In addition to this, telomeres also play an import role in regulating cell ageing (Olovnikov, 1973). Human somatic cells exhibit a limited capacity for replication in culture; after a certain period of time, they become senescent. In this state, the cells no longer divide even after having been stimulated with growth factors; however, they do not die and remain metabolically active (Goldstein, 1990). Various observations support the hypothesis that a cell determines how many more times it can divide on the basis of the length of its telomeres (Allsopp *et al.*, 1992).

In summary, the telomeres consequently possess key functions in the ageing of cells, and in stabilizing the genetic material and preventing cancer.

<u>The enzyme telomerase synthesizes the telomeres</u>

As described above, organisms which possess linear chromosomes can only replicate their genome incompletely in the absence of a special protective mechanism. Most eukaryotes use a special enzyme, i.e. telomerase, for regenerating the telomere sequences. Telomerase is expressed constitutively in the single-cell organisms which have so far been investigated. On the other hand, telomerase activity has only been measured in humans in germ cells and tumour cells, whereas neighbouring somatic tissue did not contain any telomerase (Kim *et al.*, 1994).

Telomerase can also be designated functionally as terminal telomere transferase, which is located in the cell nucleus as a multiprotein complex. While the RNA moiety of human telomerase has been known for a relatively long period of time (Feng *et al.*, 1995), the catalytic subunit of this enzyme group was recently identified in a variety of organisms (Lingner *et al.*, 1997; cf. our application PCT EP/98/03468 which is likewise pending). These catalytic subunits of telomerase are strikingly homologous both among themselves and in relation to all previously known reverse transcriptases.

WO 98/14592 also describes nucleic acid and amino acid sequences of the catalytic telomerase subunit.

Activation of telomerase in human tumours

It was originally only possible to demonstrate telomerase activity in humans in germ line cells and not in normal somatic cells (Hastie *et al.*, 1990; Kim *et al.*, 1994). Following the development of a more sensitive detection method (Kim *et al.*, 1994), a low telomerase activity was also detected in hematopoietic cells (Broccoli *et al.*, 1995; Counter *et al.*, 1995; Hiyama *et al.*, 1995). It is true, however, that these cells nevertheless exhibited a reduction in the telomeres (Vaziri *et al.*, 1994; Counter *et al.*, 1995). It has still not been resolved whether the quantity of enzyme in these cells is not sufficient for compensating the telomere loss or whether the telomerase activity which is measured stems from a subpopulation, e.g. incompletely differentiated $CD34^{+}38^{+}$ precursor cells (Hiyama *et al.*, 1995). In order to resolve this, it would be necessary to detect telomerase activity in a single cell.

Interestingly, however, significant telomerase activity was detected in a large number of the tumour tissues which had thus far been tested (1734/2031, 85%; Shay, 1997), whereas no activity was found in normal somatic tissue (1/196, <1%, Shay, 1997). In addition various investigations have shown that the telomeres still shrank in senescent cells which were transformed with viral oncoproteins and it was only possible to detect telomerase in the subpopulation which survived the growth crisis (Counter *et al.*, 1992). The telomeres were also stable in these immortalized cells. (Counter *et al.*, 1992). Similar findings from investigations in mice (Blasco *et al.*, 1996) support the assumption that reactivation of the telomerase is a late event in tumorigenesis.

Based on these results, a "telomerase hypothesis" was developed which links the loss of telomere sequences and cell ageing with telomerase activity and the development of cancer. In long-lived species such as humans, the shrinking of the telomeres can be regarded as being a mechanism for suppressing tumours. Differentiated cells which do not contain any telomerase cease their cell division at a particular telomere length. If such a cell mutates, it can only form a tumour if the cell can extend its telomeres.

Otherwise, the cell would continue to lose telomere sequences until its chromosomes became unstable and it was finally destroyed. Telomerase reactivation is presumably the main mechanism used by tumour cells to stabilize their telomeres.

It follows from these observations and considerations that it should be possible to treat tumours by inhibiting the telomerase. Conventional cancer therapies using cytostatic agents or short-wave radiation damage all the dividing cells in the body in addition to the tumour cells. However, since only germ line cells, apart from tumour cells, contain significant telomerase activity, telomerase inhibitors would attack the tumour cells more specifically and consequently elicit fewer undesirable side effects. Telomerase activity has been detected in all the tumour tissues which have so far been tested, which means that these therapeutic agents could be employed against all types of cancer. The effect of telomerase inhibitors would then set in when the telomeres of the cells had shortened to such an extent that the genome became unstable. Since tumour cells usually possess telomeres which are shorter than those of normal somatic cells, cancer cells would be the first to be eliminated by the telomerase inhibitors. By contrast, cells possessing long telomeres, such as the germ cells, would only be damaged at a much later date. Telomerase inhibitors consequently represent a potential way forward in the treatment of cancer.

It becomes possible to obtain unambiguous answers to the question of the nature and points of attack of physiological telomerase inhibitors once the manner in which expression of the telomerase gene is regulated has also been identified.

Regulation of gene expression in eukaryotes

There are a large number of points in eukaryotic gene expression, i.e. the cellular flow of information from the DNA to the protein by way of the RNA, at which regulatory mechanisms can exert an effect. Examples of individual control steps are gene amplification, the recombination of gene loci, chromatin structure, DNA methylation, transcription, post-transcriptional modifications of mRNA, mRNA transport, translation and post-translational modifications of proteins. Studies which

have been carried out to date indicate that control at the level of transcription initiation is of the greatest importance (Latchman, 1991).

A region which is responsible for regulating transcription, and which is designated the promoter region, is located directly upstream of the transcription start of a gene which is transcribed by RNA polymerase II. Comparison of the nucleotide sequences of promoter regions from a large number of known genes shows that particular sequence motifs occur regularly in this region. These elements include, inter alia, the TATA box, the CCAAT box and the GC box, which elements are recognized by specific proteins. The TATA box, which is located about 30 nucleotides upstream of the transcription start, is, for example, recognized by the TFIID subunit TBP ("TATA box-binding protein"), whereas particular GC-rich sequence segments are specifically bound by the transcription factor Sp1 ("specificity protein1").

The promoter can be functionally subdivided into a regulatory segment and a constitutive segment (Latchman, 1991). The constitutive control region comprises the so-called core promoter which enables transcription to be initiated correctly. This promoter contains the sequence elements which are described as UPE's (upstream promoter elements) which are necessary for efficient transcription. The regulatory control segments, which can be interlaced with the UPE's, possess sequence elements which can be involved in the signal-dependent regulation of transcription by hormones, growth factors, etc. They impart tissue-specific or cell-specific promoter properties.

DNA segments which are able to exert an influence on gene expression over relatively large distances are a characteristic feature of eukaryotic genes. These elements can be located upstream or downstream of a transcription unit, or within the unit, and can perform their function independently of their orientation. These sequence segments may reinforce (enhancers) or attenuate (silencers) promoter activity. In a similar way to the promoter regions, enhancers and silencers also accommodate several binding sites for transcription factors.

The invention relates to the DNA sequences from the 5′-flanking region of the gene for the catalytically active human telomerase subunit and intron sequences for this gene.

The invention particularly relates to the 5′-flanking regulatory DNA sequence which contains the promoter DNA sequence for the gene for the human catalytic telomerase subunit, as depicted in Fig. 10 (SEQ ID NO 3).

The invention furthermore relates to part regions of the 5'-flanking regulatory DNA sequence, as depicted in Fig. 4 (SEQ ID NO 1), which has a regulatory effect.

Intron sequences for the gene for the human catalytic telomerase subunit, in particular those sequences which have a regulatory effect, are also part of the subject-matter of the present invention. The intron sequences according to the invention are described in detail in the context of Example 5 (cf. SEQ ID NO 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20).

The invention furthermore relates to a recombinant construct which comprises the DNA sequences according to the invention, in particular the 5'-flanking DNA sequence of the gene for the human catalytic telomerase subunit, or part regions thereof.

Preference is given to recombinant constructs which, in addition to the DNA sequences according to the invention, in particular the 5'-flanking DNA sequence of the gene for the human catalytic telomerase subunit, or part regions thereof, also contain one or more additional DNA sequences which encode polypeptides or proteins.

According to a particularly preferred embodiment, these additional DNA sequences encode antineoplastic proteins.

Particular preference is given to those antineoplastic proteins which inhibit angiogenesis directly or indirectly. Examples of these proteins are:

Plasminogen activator inhibitor (PAI-1), PAI-2, PAI-3, angiostatin, endostatin, platelet factor 4, TIMP-1, TIMP-2, TIMP-3 and leukaemia inhibitory factor (LIF).

Antineoplastic proteins which have a direct or indirect cytostatic effect on tumours are likewise particularly preferred. These proteins include, in particular:

perforin, granzyme, IL-2, IL-4, IL-12, interferons, such as IFN-α, IFN-ß and IFN-γ, TNF, TNF-α, TNF-ß, oncostatin M; tumour suppressor genes, such as p53, retinoblastoma.

Particular preference is furthermore given to antineoplastic proteins which, where appropriate in addition to their antineoplastic effect, stimulate inflammations and thereby contribute to the elimination of tumour cells. Examples of these proteins are:

RANTES, monocyte chemotactic and activating factor (MCAF), IL-8, macrophage inflammatory protein (MIP-1α,-ß), neutrophil activating protein-2 (NAP-2), IL-3, IL-5, human leukaemia inhibitory factor (LIF), IL-7, IL-11, IL-13, GM-CSF, G-CSF and M-CSF.

Particular preference is furthermore given to antineoplastic proteins which, due to their action as enzymes, are able to convert precursors of an antineoplastic active compound into an antineoplastic active compound. Examples of these enzymes are:

herpes simplex virus thymidine kinase, varicella zoster virus thymidine kinase, bacterial nitroreductase, bacterial ß-glucuronidase, plant ß-glucuronidase from *Secale cereale*, human glucuronidase, human carboxypeptidase, bacterial carboxypeptidase, bacterial ß-lactamase, bacterial cytosine deaminidase, human catalase and/or phosphatase, human alkaline phosphatase, type 5 acid phosphatase, human

lysooxidase, human acid D-aminooxidase, human glutathione peroxidase, human eosinophil peroxidase and human thyroid peroxidase.

The abovementioned recombinant constructs can also contain DNA sequences which encode factor VIII or factor IX, or part fragments thereof. These DNA sequences also include other blood clotting factors.

The abovementioned recombinant constructs can also contain DNA sequences which encode a reporter protein. Examples of these reporter proteins are:

Chloramphenicol acetyl transferase (CAT), glow-worm luciferase (LUC), ß-galactosidase (ß-Gal), secreted alkaline phosphatase (SEAP), human growth hormone (hGH), ß-glucuronidase (GUS), green-fluorescing protein (GFP), and all the variants derived therefrom, aquarin and obelin.

Recombinant constructs according to the invention can also contain DNA which encodes the human catalytic telomerase subunit and its variants and fragments in the antisense orientation. Where appropriate, these constructs can also contain other protein subunits of the human telomerase and the telomerase RNA component in the antisense orientation.

The recombinant constructs can, in addition to the DNA which encodes the human catalytic telomerase subunit, and its variants and fragments, also contain other protein subunits of the human telomerase and the telomerase RNA component.

The invention furthermore relates to a vector which contains the abovementioned DNA sequences according to the invention, in particular the 5'-flanking DNA sequences and also one or more of the other DNA sequences mentioned above.

The preferred vector for these constructs is a virus, for example a retrovirus, an adenovirus, an adeno-associated virus, a herpes simplex virus, a vaccina virus, a lentiviral virus, a Sindbis virus and a Semliki forest virus.

Preference is also given to using plasmids as vectors.

The invention furthermore relates to pharmaceutical preparations which comprise recombinant constructs or vectors according to the invention; for example a preparation in a colloidal dispersion system.

Examples of suitable colloidal dispersion systems are liposomes or polylysine ligands.

The preparations of the constructs or vectors according to the invention in colloidal dispersion systems can be supplemented with a ligand which binds to the membrane structures of tumour cells. Such a ligand can, for example, be attached to the construct or the vector or else be a component of the liposome structure.

Suitable ligands are, in particular, polyclonal or monoclonal antibodies, or antibody fragments thereof, which bind, by their variable domains, to the membrane structures of tumour cells, or substances carrying mannose terminally, cytokines or growth factors, or fragments or part sequences thereof, which bind to receptors on tumour cells.

Examples of corresponding membrane structures are receptors for a cytokine or a growth factor, such as IL-1, EGF, PDGF, VEGF, TGF ß, insulin or insulin-like growth factor (ILGF), or adhesion molecules, such as SLeX, LFA-1, MAC-1, LECAM-1 or VLA-4, or the mannose-6-phosphate receptor.

The present invention includes pharmaceutical preparations which, in addition to the vector constructs according to the invention, can also comprise non-toxic, inert, pharmaceutically suitable excipients. It is possible to conceive of administering (e.g. intravenously, intraarterially, intramuscularly, subcutaneously, intradermally, anally, vaginally, nasally, transdermally, intraperitoneally, as an aerosol or orally) these preparations at the site of a tumour or administering them systemically.

The vector constructs according to the invention can be employed in gene therapy.

The invention furthermore relates to a recombinant host cell, in particular a recombinant eukaryotic host cell, which harbours the above-described constructs or vectors.

The invention furthermore relates to a process for identifying substances which affect the promoter activity, silencer activity or enhancer activity of the catalytic telomerase subunit, with this process comprising the following steps:

A. adding a candidate substance to a host cell which harbours the regulatory DNA sequence according to the invention, in particular the 5'-flanking regulatory DNA sequence for the gene for the human catalytic telomerase subunit, or a part region thereof which has a regulatory effect, which sequence or part region is functionally linked to a reporter gene, and

B. measuring the effect of the substance on expression of the reporter gene.

The process can be employed for identifying substances which increase the promoter activity, silencer activity or enhancer activity of the catalytic telomerase subunit.

The process can furthermore be employed for identifying substances which inhibit the promoter activity, silencer activity or enhancer activator of the catalytic telomerase subunit.

The invention furthermore relates to a process for identifying factors which bind specifically to fragments of the DNA fragments according to the invention, in particular the 5'-flanking regulatory DNA sequence of the catalytic telomerase subunit. This method comprises screening an expression cDNA library using the above-described DNA sequence, or subfragments of widely differing length, as the probe.

The above-described constructs or vectors can also be used for preparing transgenic animals.

The invention furthermore relates to a process for detecting telomerase-associated conditions in a patient, which process comprises the following steps:

A. incubating a construct or vector, which contains the DNA sequence according to the invention, in particular the 5'-flanking regulatory DNA sequence for the gene for the human catalytic telomerase subunit, or a part region thereof having a regulatory effect, and a reporter gene, with body fluids or cell samples,

B. detecting the activity of the reporter gene in order to obtain a diagnostic value; and

C. comparing the diagnosic value with standard values for the reporter gene construct in standardized normal cells or body fluids of the same type as the test sample;

The detection of diagnostic values which are higher or lower than the standard comparative values indicates a telomerase-associated condition, which in turn indicates a pathogenic condition.

Explanation of the figures:

Fig. 1: Southern blot analysis using genomic DNA from various species

A: Photograph of an ethidium bromide-stained 0.7% agarose gel containing approximately 4 µg of Eco RI-cut genomic DNA. Track 1 contains Hind III-cut λ DNA as size markers (23.5, 9.4, 6.7, 4.4, 2.3, 2.0 and 0.6 kb). Tracks 2 to 10 contain human, rhesus monkey, Sprague

Dawley rat, BALB/c mouse, dog, bovine, rabbit, chicken and yeast (*Saccharomyces cerevisiae*) genomic DNA.

B: Autoradiogram, corresponding to Fig.1 A, of a Southern blot analysis in which radioactively labelled hTC-cDNA probe of about 720 bp in length is used for the hybridization.

Fig. 2: Restriction analysis of the recombinant λ DNA of the phage clone P12, which hybridizes with a probe from the 5' region of the hTC cDNA.

The figure shows a photograph of an ethidium bromide-stained 0.4% agarose gel. Tracks 1 and 2 contain Eco RI/Hind III-cut λ DNA and a 1 kb ladder from Gibco as size markers. Tracks 3 - 7 each contain 250 ng of the DNA from the recombinant phage which has been cut with Bam HI (track 3), Eco RI (track 4), Sal I (track 5), Xho I (track 6) and Sac I (track 7). The arrows mark the two λ arms of the vector EMBL3 Sp6/T7.

Fig. 3: Restriction analysis and Southern blot analysis of the recombinant λ DNA of the phage clone which hybridizes with a probe from the 5' region of the hTC cDNA.

A: The figure shows a photograph of an ethidium bromide-stained 0.8% agarose gel. Tracks 1 and 15 contain a 1 kb ladder from Gibco as size markers. Tracks 2 to 14 each contain 250 ng of cut λ DNA from the recombinant phage clone. The following enzymes were employed: track 2: Sac I, track 3: Xho I, track 4: Xho I, Xba I, track 5: Sac I, Xho I, track 6: Sal I, Xho I, Xba I, track 7: Sac I, Xho I, Xba I, track 8: Sac I, Sal I, Xba I, track 9: Sac I, Sal I, BamH I, track 10: Sac I, Sal I, Xho I, track 11: Not I, track 12: Sma I, track 13: empty, track 14: not digested.

B: Autoradiogram, corresponding to Fig. 3 A, of a Southern blot analysis. A 5'-hTC cDNA fragment of about 420 bp in length was used as the probe for the hybridization.

Fig. 4: Partial DNA sequence of the 5'-flanking region and of the promoter of the gene for the human catalytic telomerase subunit. The ATG start codon in the sequence is printed in bold. The depicted sequence corresponds to SEQ ID NO 1.

Fig. 5: Use of primer extension analysis to identify the transcription start.

The figure shows an autoradiogram of a denaturing polyacrylamide gel which was selected for depicting a primer extension analysis. An oligonucleotide having the sequence 5′GTTAAGTTGTAGCTTACACTGGTTCTC 3′ was used as the primer. The primer extension reaction was loaded in track 1. Tracks G, A, T and C constitute the sequence reactions using the same primer and the corresponding dideoxynucleotides. The thick arrow marks the main transcription start while the thin arrows point to three subsidiary transcription start points.

Fig. 6: cDNA sequence of the human catalytic telomerase subunit (hTC; cf. our pending application PCT/EP/98/03468). The depicted sequence corresponds to SEQ ID NO 2.

Fig. 7: Structural organization and restriction map of the human hTC gene and its 5′-flanking and 3′-flanking regions.

Exons are shown as consecutively numbered rectangles which are filled-in in black, and introns are shown as regions which are not filled in. Untranslated sequence segments in the exons are hatched. Translation starts in exon 1 and ends in exon 16. Restriction enzyme cleavage sites

are marked as follows: S, SacI; X, XhoI. The relative arrangement of the five phage clones (P2, P3, P5, P12, P17), and of the product from the genome walking, are shown by thin lines. As the dots indicate, the sequence of intron 16 has only been partly deciphered.

Fig. 8: HTL splice variants.

A: Diagrammatic structure of the hTC mRNA splice variants. The complete hTC mRNA is depicted as a rectangle with a grey background in the upper region of the figure. The 16 exons are depicted in accordance with their size. The translation start (ATG) and the stop codon, and also the telomerase-specific T motif, and the seven RT motifs, are all shown. The hTC variants are subdivided into deletion and insertion variants. The missing exon sequences are marked in the deletions. The insertions are shown by additional white rectangles. The sizes and origins of the inserted sequences are given. Newly formed stop codons are marked. The size of the insertion in variant INS2 is unknown.

B: Exon-intron transitions in the hTC splice variants. Unspliced 5′-flanking and 3′-flanking sequences are shown as white rectangles. The origins of the exon and intron sequences are given. Intron and exon sequences are shown in small letters and large letters, respectively. The donor and acceptor sequences in the splice sites are underlaid as grey rectangles, and their exon and intron origins are also given.

Fig. 9: Identification of the transcription start by means of RT-PCR analysis.

The RT-PCR was carried out using a cDNA library prepared from HL 60 cells and genomic DNA as the positive control. A common 3' primer hybridizes to a region of the exon 1 sequence. The positions of the different 5' primers in the coding region or the 5'-flanking region are given. In the negative control, no template DNA was added to the PCR reaction. M: DNA size marker.

Fig. 10: Nucleotide sequence and structural features of the hTC promoter.

The figure depicts 11273 bp of the 5′-flanking hTC gene sequence, beginning with the translation start codon ATG (+1). The putative region of the translation start is underlined. Possible regulatory sequence segments within the 4000 bp upstream of the translation start are ringed. The depicted sequence corresponds to SEQ ID NO 3.

Fig. 11: Activity of the hTC promoter in HEK-293 cells.

The first 5000 bp of the 5'-flanking hTC gene region are shown diagrammatically in the upper part of the figure. The ATG start codon is picked out. CpG-rich islands are marked by grey rectangles. The sizes of the hTC promoter-luciferase construct are shown on the left-hand side of the figure. The promoterless pGL2 basic construct and the SV40 promoter construct pGL2-Pro were used as controls in each transfection. The relative luciferase activities of the different promoter constructs in HEK cells are shown as continuous bars on the right-hand side of the figure. The standard deviation is indicated. The numerical values represent the average of two independent experiments which were carried out in duplicate.

Tab. 1: Exon-intron transitions in the hTC gene

The table lists the nucleotide sequences at the 3′ and 5′ splice transitions of the hTC gene. The consensus sequences for donor and acceptor sequences (AG and GT) are underlaid with grey rectangles. The table shows the intron sequences (small letters) and exon sequences (large letters) which flank the splice acceptor and donor sites. The sizes of the exons and introns are given in bp.

Tab. 2: Potential binding sites for DNA-binding factors in the nucleotide sequence of intron 2

The search for possible DNA-binding factors (e.g. transcription factors) was carried out using the "find pattern" algorithm from the Genetics Computer Group (Madison, USA) GCG sequence analysis program package. The table lists the abbreviations of the DNA-binding factors which were identified and their location in intron 2.

Tab. 1

3' Acceptor Sequence				5' Donor Sequence			
Intron	Exon	Exon No.	bp	Exon	Intron	Intron No.	bp
5' flanking region	GTTTCAGGCAGCGCTGCGT	1	281	CGCCCCCTCCTTCCGCCAG	gtgggcctccccggggtcg	1	104
cagggcgcttccccgcag	GTGTCCTGCCTGAAGGAGC	2	1354	TGGCTGCGCAGGAGCCCAG	gtgaggaggtggtggccgt	2	8616
catgtccttctcgtttaag	GGGTTGGCTGTGTTCCGGC	3	196	TGCAAAGCATTGGAATCAG	gtactgtatccccacgcca	3	2089
gaggggctctctattgcag	ACAGCACTTGAAGAGGGTG	4	181	GTTCCGCAGAGAAAAGAGG	gtggctgtgctttggttta	4	687
cccatgctgtccccgcccag	GCCGAGCGTCTCACCTCGA	5	180	TGAGCTGTACTTTGTCAAG	gtgggtgccggggaccccc	5	494
ctcgcctccactcacacag	GTGGATGTGACGGGCGCGT	6	156	CAAGGCCTTCAAGAGCCAC	gtaaggttcacgtgtgata	6	>4660
ccctctcctctgccggcag	GTCTCTACCTTGACAGACC	7	96	TGCCGTCGTCATCGAGCAG	gtctgggcactgccctgca	7	980
ctcccgtctgctttcgcag	AGCTCCTCCCTGAATGAGG	8	86	CCGTGCGCATCAGGGGCAA	gtgagtcaggtggccaggt	8	2485
ctgtgtcttcccgccccag	GTCCTACGTCCAGTGCCAG	9	114	CGGGGATTCGGCGGGACGG	gtgaggcctcctcttcccc	9	1984
gtattttcccttattttag	GCTGCTCCTGCGTTTGGTG	10	72	ACGCGAAAACCTTCCTCAG	gtgaggcccgtgccgtgtg	10	1871
cattgcccctctgccttag	GACCCTGGTCCGAGGTGTC	11	189	TGCAGAGCGACTACTCCAG	gtgagcgcacctggccgga	11	3801
attccccctgtgtctcag	CTATGCCCGGACCTCCATC	12	127	CCTGTTTCTGGATTTGCAG	gtgagcaggctgatggtca	12	880
tctttcttggcgactctag	GTGAACAGCCTCCAGACGG	13	62	TCCTGCTGCAGGCGTACAG	gtgagccgccaccaagggg	13	3187
ctgtccgccatcctctcag	GTTTCACGCATGTGTGCTG	14	125	CTGAAAGCCAAGAACGCAG	gtatgtgcaggtgcctggc	14	781
agcctctgttttccccag	GGATGTCGCTGGGGGCCAA	15	138	CTGGGGTCACTCAGGACAG	gcaagtgtgggtggaggcc	15	536
tctgattttggccccgcag	CCCAGACGCAGCTGAGTCG	16	664	TTTTTCAGTTTTGAAAAAA	3' flanking region		

Tab. 2

Factors	**Location in intron 2**
C/EBP	2925
CRE.2	2749
Sp1	2378, 4094, 4526, 4787, 4835, 4995
AP-2 CS3	5099
AP-2 CS4	2213, 3699, 4667, 5878, 5938, 6059, 6180, 6496
AP-2 CS5	5350, 5798, 5880, 5940, 6061, 6182, 6375, 6498
PEA3	934, 2505
P53	2125
GR uteroglobin	848, 1487, 2956
PR uteroglobin	3331
Zeste-white	1577, 1619, 1703, 1745, 1787, 1829, 1871, 1913, 1955, 1997, 2039, 2081, 3518, 3709, 4765, 5014, 5055
GRE	846
MyoD-MCK right site/rev	447, 509, 558, 1370, 1595, 1900, 2028, 2099, 4557
MyoD-MCK left site	108, 118, 453, 1566, 1608, 1692, 1734, 1818, 1902, 1986, 2372, 2460, 2720, 3491, 5030
Ets-1 CS	6408
AP1	3784, 4406
CREB	2801
GATA-1	839, 1390, 3154
c-Myc	108, 118, 453, 1566, 1608, 1692, 1734, 1818, 1902, 1986, 2372, 2460, 2720, 3491, 5030
CACCC site	991
CCAAT site	1224
CCAC box	992
CAAT site	463, 2395
Rb site	992, 4663
TATA	3650
CDEI	106, 1564, 1606, 1690, 1732, 1816, 1900, 1984

Examples

The human gene for the catalytic telomerase subunit (ghTC), and the regions of this gene located 5' and 3', were cloned, while the start point for transcription was determined, potential binding sites for DNA-binding proteins were identified and active promoter fragments were highlighted. The sequence of the hTC cDNA (Fig. 6) has already been reported in our application PCT/EP/98/03468, which is also pending. Unless otherwise mentioned, all the data refer to the position of the cDNA in this sequence.

Example 1

A genomic Southern blot analysis was used to determine whether ghTC constitutes a single gene in the human genome or whether there exist several loci for the hTC gene and possibly also ghTC pseudogenes.

In order to do this, a commercially available zoo blot from Clontech was subjected to Southern blot analysis. This blot contains 4 µg of Eco RI-cut genomic DNA from nine different species (human, monkey, rat, mouse, dog, bovine, rabbit, chicken and yeast). With the exception of yeast, chicken and human, the DNA was isolated from kidney tissue. The human genomic DNA was isolated from placenta and the chicken genomic DNA was purified from liver tissue. An hTC cDNA fragment of about 720 bp in length, which was isolated from hTC cDNA, variant Del2 (position 1685 to 2349 plus 2531 to 2590 in Fig. 6 [deletion 2; cf. Example 5 in Fig. 8]), was used as the radioactively labelled probe in the autoradiogram in Fig. 1. The experimental conditions for the blot hybridization and washing steps were taken from Ausubel *et al.* (1987).

In the case of the human DNA, the probe recognizes two specific DNA fragments. The smaller Eco RI fragment, of from about 1.5 to 1.8 kb in length, probably originates from two Eco RI cleavage sites in an intron in the ghTC DNA. On the

basis of this result, it is to be assumed that only one single ghTC gene is present in the human genome.

Example 2

In order to isolate the 5' flanking hTC gene sequence, approx. 1.5×10^6 phages from a human genomic placenta gene library (EMBL 3 SP6/T7 from Clontech, order number HL1067j) were hybridized on nitrocellulose filters (0.45 µm; from Schleicher and Schuell), in accordance with the manufacturer's instructions, with a radioactively labelled 5′-hTC cDNA fragment of about 500 bp in length (position 839 to 1345 in Fig. 6). The nitrocellulose filters were firstly incubated, at 42°C for two hours, in 2 x SSC (0.3 M NaCl; 0.5 M Tris-HCl, pH 8.0) and then in a prehybridization solution (50% formamide; 5 x SSPE, pH 7.4; 5 x Denhard's solution; 0.25% SDS; 100 µg of herring sperm DNA/ml). For the overnight hybridization, the prehybridization solution was supplemented with 1.5×10^6 cpm of denatured, radioactively labelled probe/ml of solution. Nonspecifically bound radioactive DNA was removed under stringent conditions, i.e. by means of three five-minute steps of washing with 2 x SSC; 0.1% SDS at from 55 to 65°C. The filters were evaluated by autoradiography.

The phage clones which were identified in this primary investigation were purified (Ausubel *et al.* (1987)). In subsequent analyses, one phage clone, i.e. P12 turned out to be potentially positive. A λ DNA preparation carried out on this phage (Ausubel *et al.* (1987)), and the subsequent restriction digestion with enzymes which release the genomic insert in fragments, showed that this phage clone contains an insert of approx. 15 kb in the vector (Fig. 2).

In order to isolate the complete hTC gene sequence, in each case from 1 to 1.5×10^6 phages were screened, in independent experiments, with in each case different radioactively labelled probes, as described above.

The phage clones which were identified in these primary investigations, and which were positive for the corresponding probes, were purified. The phage clone P17 was found to contain an hTC cDNA fragment of about 250 bp in length (position 1787 to 2040 in Fig. 6). The phage clone P2 was identified as containing an hTC cDNA fragment of about 740 bp in length (position 1685 to 2349 plus 2531 to 2607 in Fig. 6 [deletion 2; cf. Example 5]). The phage clones P3 and P5 were found to contain a 3′ hTC cDNA fragment of 420 bp in length (position 3047 to 3470 in Fig. 6). After the λ DNA had been prepared from these phages, and subsequently subjected to restriction digestion with enzymes which release the genomic insert in fragments, the inserts were subcloned into plasmids (Example 4).

Example 3

In order to investigate whether the 5′ end of the hTC cDNA was also present in the insert in the recombinant phage clone P12, the λ DNA from this clone was hybridized, in a Southern blot analysis, with a radiactively labelled hTC cDNA fragment of about 440 bp in length (position 1 to 440 in Fig. 6) from the extreme 5′ region (Fig. 3).

Since the isolated λ DNA from the positive clone also hybridizes with the extreme 5' end of the hTC cDNA, this phage probably also contains the 5' sequence region flanking the ATG start codon.

Example 4

In order to subclone the entire 15 kb insert in the positive phage clone P12 in the form of subfragments, and subsequently to sequence these fragments, restriction endonucleases which, on the one hand, release the entire insert from EMBL3 Sp6/T7 (cf. Example 2) and, in addition, cut within the insert, were selected for digesting the DNA.

In all, two Xho I subfragments, of about 8.3 and about 6.5 kb in length, respectively, and three Sac I subfragments, of about 8.5, about 3.5 and about 3 kb in length, respectively, were subcloned into the pBluescript KS(+) vector (from Stratagene). The 5123 bp 5′-flanking nucleotide sequence of the ghTC gene region, starting from the ATG start codon, was determined by analysing the sequences of these fragments (Fig. 4; corresponding to SEQ ID NO 1). Fig. 4 depicts the first 5123 bp (starting from the ATG start codon). Fig. 10 depicts the entire cloned 5' sequence (corresponding to SEQ ID NO 3).

In order to subclone the entire insert, of approx. 14.6 kb in size, in phage clone P17 in the form of subfragments, restriction endonucleases which, on the one hand, release the entire insert from EMLB3 Sp6/T7 and, in addition, cut a few times within the insert, were selected for digesting the DNA. Three XhoI/BamHI fragments, of 7.1 kb, 4.2 kb and 1.5 kb in size, respectively, and one BamHI fragment, of 1.8 kb in size, were subcloned by means of using a combination digestion with the enzymes XhoI and BamHI. Combination restriction digestion with the enzymes XhoI and XbaI resulted in a XhoI/XbaI fragment of 6.5 kb in size, and two XhoI fragments, of 6.5 kb and 1.5 kb in size, respectively, being cloned.

Digestion with the restriction enzyme XhoI was used to subclone the insert, of approx. 17.9 kb in size, in phage clone P2 in the form of subfragments. In all, three XhoI subfragments, of 7.5 kb, 6.4 kb and 1.6 kb in length, respectively, were cloned. Four SacI fragments, of 4.8 kb, 3 kb, 2 kb and 1.8 kb in size, respectively, were additionally subcloned by digesting with the restriction enzyme SacI.

The insert, of approx. 13.5 kb in size, in phage clone P3 was subcloned by digesting with the restriction enzymes SacI and/or XhoI. Six SacI subfragments, of 3.2 kb, 2 kb, 0.9 kb, 0.8 kb, 0.65 kb and 0.5 kb in length, respectively, and two XhoI subfragments, of 6.5 kb and 4.3 kb in length, respectively, were obtained in this connection.

The insert, of approx. 13.2 kb in size, in phage clone P5 was subcloned by digesting with the restriction enzymes SacI and/or XhoI. In all, SacI fragments of 6.5 kb, 3.3 kb, 3.2 kb, 0.8 kb and 0.3 kb in size, and XhoI fragmente of 7 kb and 3.2 kb in size, were subcloned.

In order to clone the hTC genomic sequence region located 3' of phage clone P17 and 5' of phage clone P2, 3 genomic walkings were carried out using the Clontech GenomeWalker™ kits (catalogue number K1803-1) and various combinations of primers. In a final volume of 50 µl, 10 pmol of dNTP mix were added to 1 µl of human GenomeWalker Library HDL (from Clontech), and a PCR reaction was carried out in 1xKlen Taq PCR reaction buffer and 1xAdvantage Klen Taq polymerase mix (from Clontech). 10 pmol of an internal gene-specific primer, and 10 pmol of the adaptor primer AP1 (5'-GTAATACGACTCACTATAGGGC-3'; from Clontech) were added as primers. The PCR was carried out in 3 steps as a touchdown PCR. First of all, denaturation was carried out at 94°C for 20 sec, and the primers were then annealed, and the DNA chain extended, at 72°C for 4 min, over 7 cycles. There then followed 37 cycles in which the DNA was denaturated at 94°C for 20 sec but the subsequent primer extension took place at 67°C for 4 min. In conclusion, there followed a chain extension at 67°C for 4 min. After this first PCR, the PCR product was diluted 1:50. One µl of this dilution was used in a second nested PCR together with 10 pmol of dNTP mix in 1xKlen Taq PCR reaction buffer and 1xAdvantage Klen Taq polymerase mix and also 10 pmol of a nested gene-specific primer and 10 pmol of the nested Marathon Adaptor primers AP2 (5'-ACTATAGGGCACGCGTGGT-3'; from Clontech). The PCR conditions corresponded to the parameters which were selected in the first PCR. As the sole exception, only 5 cycles rather than 7 cycles were selected in the first PCR step and only 24 cycles, instead of 37 cycles, were run in the second PCR step. The products of this nested genomic walking PCR were cloned into the TA Cloning Vector pCRII from InVitrogen.

In the first genomic walking, the gene-specific primer C3K2-GSP1 (5'-GACGTGGCTCTTGAAGGCCTTG-3') and the nested gene-specific primer C3K2-GSP2 (5'-GCCTTCTGGACCACGGCATACC-3') were used, together with the HDL library 4, and a PCR fragment of 1639 bp in length was obtained. In the second genomic walking, a PCR fragment of 685 bp in length was amplified from the HDL library 4 using the gene-specific primer C3F2 (5'-CGTAGTTGAGCACGCTGAACAGTG-3') and the nested gene-specific primer C3F (5'-CCTTCACCCTCGAGGTGAGACGCT-3. The third genomic walking mixture, using the gene-specific primer DEL5-GSP1 (5'-GGTGGATGTGACGGGCGCGTACG-3') and the nested gene-specific primer C5K-GSP1 (5'-GGTATGCCGTGGTCCAGAAGGC-3'), led to a 924 bp PCR fragments being cloned from the HDL library 1. In all, 2100 bp of the genomic hTC region located 3' of phage clone P17 were identified using this genomic walking method (see Fig. 7).

The subcloned fragments, and the genomic walking products, were sequenced in single-stranded form. The Lasergene Biocomputing Software (DNASTAR Inc. Madison, Wisconsin, USA) was used to identify overlapping regions and form contigs. In all, 2 large contigs were assembled from the sequences collected from phage clones P12, P17, P2, P3 and P5, and also the sequence data from the genomic walking. Contig 1 consists of sequence data from phage clones P12 and P17 and the sequence data from the genomic walking. Contig 2 was put together from the sequences from phage clones P2, P3 and P5. Overlapping phage clone regions are shown diagrammaticaly in Fig. 7. The sequence data from the 2 contigs are shown below. The ATG start codon in contig 1 is underlined. The TGA stop codon is underlined in contig 2.

Contig1:

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ACTTGAGCCC AAGAGTTCAA GGCTACGGTG AGCCATGATT GCAACACCAC ACGCCAGCCT TGGTGACAGA 70
ATGAGACCCT GTCTCAAAAA AAAAAAAAAA AATTGAAATA ATATAAAGCA TCTTCTCTGG CCACAGTGGA 140
ACAAAACCAG AAATCAACAA CAAGAGGAAT TTTGAAAACT ATACAAACAC ATGAAAATTA AACAATATAC 210
TTCTGAATGA CCAGTGAGTC AATGAAGAAA TTAAAAAGGA AATTGAAAAA TTTATTTAAG CAAATGATAA 280
CGGAAACATA ACCTCTCAAA ACCCACGGTA TACAGCAAAA GCAGTGCTAA GAAGGAAGTT TATAGCTATA 350
AGCAGCTACA TCAAAAAAGT AGAAAAGCCA GGCGCAGTGG CTCATGCCTG TAATCCCAGC ACTTTGGGAG 420
GCCAAGGCGG GCAGATCGCC TGAGGTCAGG AGTTCGAGAC CAGCCTGACC AACACAGAGA AACCTTGTCG 490
CTACTAAAAA TACAAAATTA GCTGGGCATG GTGGCACATG CCTGTAATCC CAGCTACTCG GGAGGCTGAG 560
GCAGGATAAC CGCTTGAACC CAGGAGGTGG AGGTTGCGGT GAGCCGGGAT TGCGCCATTG GACTCCAGCC 630
TGGGTAACAA GAGTGAAACC CTGTCTCAAG AAAAAAAAAA AAGTAGAAAA ACTTAAAAAT ACAACCTAAT 700
GATGCACCTT AAAGAACTAG AAAAGCAAGA GCAAACTAAA CCTAAAATTG GTAAAAGAAA AGAAATAATA 770
AAGATCAGAG CAGAAATAAA TGAAACTGAA AGATAACAAT ACAAAAGATC AACAAAATTA AAAGTTGGTT 840
TTTTGAAAAG ATAAACAAAA TTGACAAACC TTTGCCCAGA CTAAGAAAAA AGGAAAGAAG ACCTAAATAA 910
ATAAAGTCAG AGATGAAAAA AGAGACATTA CAACTGATAC CACAGAAATT CAAAGGATCA CTAGAGGCTA 980
CTATGAGCAA CTGTACACTA ATAAATTGAA AAACCTAGAA AAAATAGATA AATTCCTAGA TGCATACAAC 1050
CTACCAAGAT TGAACCATGA AGAAATCCAA AGCCCAAACA GACCAATAAC AATAATGGGA TTAAAGCCAT 1120
AATAAAAAGT CTCCTAGCAA AGAGAAGCCC AGGACCCAAT GGCTTCCCTG CTGGATTTTA CCAATCATTT 1190
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TCTACATGGC CAGTATTACC CTGATTCCAA AACCAGACAA AAACACATCA AAAACAAACA AACAAAAAAA 1330
CAGAAAGAAA GAAAACTACA GGCCAATATC CCTGATGAAT ACTGATACAA AAATCCTCAA CAAAACACTA 1400
GCAAACCAAA TTAAACAACA CCTTCGAAAG ATCATTCATT GTGATCAAGT GGGATTTATT CCAGGGATGG 1470
AAGGATGGTT CAACATATGC AAATCAATCA ATGTGATACA TCATCCCAAC AAAATGAAGT ACAAAAACTA 1540
TATGATTATT TCACTTTATG CAGAAAAAGC ATTTGATAAA ATTCTGCACC CTTCATGATA AAAACCCTCA 1610
AAAAACCAGG TATACAAGAA ACATACAGGC CAGGCACAGT GGCTCACACC TGCGATCCCA GCACTCTGGG 1680
AGGCCAAGGT GGGATGATTG CTTGGGCCCA GGAGTTTGAG ACTAGCCTGG GCAACAAAAT GAGACCTGGT 1750
CTACAAAAAA CTTTTTTAAA AAATTAGCCA GGCATGATGG CATATGCCTG TAGTCCCAGC TAGTCTGGAG 1820
GCTGAGGTGG GAGAATCACT TAAGCCTAGG AGGTCGAGGC TGCAGTGAGC CATGAACATG TCACTGTACT 1890
CCAGCCTAGA CAACAGAACA AGACCCCACT GAATAAGAAG AAGGAGAAGG AGAAGGGAGA AGGGAGGGAG 1960
AAGGGAGGAG GAGGAGAAGG AGGAGGTGGA GGAGAAGTGG AAGGGGAAGG GGAAGGGAAA GAGGAAGAAG 2030
AAGAAACATA TTTCAACATA ATAAAAGCCC TATATGACAG ACCGAGGTAG TATTATGAGG AAAAACTGAA 2100
AGCCTTTCCT CTAAGATCTG GAAAATGACA AGGGCCCACT TTCACCACTG TGATTCAACA TAGTACTAGA 2170
AGTCCTAGCT AGAGCAATCA GATAAGAGAA AGAAATAAAA GGCATCCAAA CTGGAAAGGA AGAAGTCAAA 2240
TTATCCTGTT TGCAGATGAT ATGATCTTAT ATCTGGAAAA GACTTAAGAC ACCACTAAAA AACTATTAGA 2310
GCTGAAATTT GGTACAGCAG GATACAAAAT CAATGTACAA AAATCAGTAG TATTTCTATA TTCCAACAGC 2380
AAACAATCTG AAAAAGAAAC CAAAAAAGCA GCTACAAATA AAATTAAACA GCTAGGAATT AACCAAAGAA 2450
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AGATATTCCA TGTTCATAGA TTGGAAGAAT AAATACTGTT AAAATGTCCA TACTACCCAA AGCAATTTAC 2590
AAATTCAATG CAATCCCTAT TAAAATACTA ATGACGTTCT TCACAGAAAT AGAAGAAACA ATTCTAAGAT 2660
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CCCCTGGG                                                                     25138
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Example 5

Comparison of the above-described genomic hTC sequence and the sequence of the hTC cDNA (Fig. 6; corresponding to SEQ ID NO 2) made it possible to elucidate the exon-intron structure of the hTC gene. The genomic organization of the hTC gene is illustrated diagrammatically in Fig. 7. The coding region of the hTC gene is composed of 16 exons which vary in size between 62 bp and 1354 bp (see Table 1). Exon 1 contains the translation start codon ATG. The translation stop codon TGA and the 3'-untranslated region lie on exon 16 (Fig. 8). No possible polyadenylation signal (AATAAA) was found either in exon 16 or in the 3195 bp of the following

3'-flanking region. The exon-intron transitions were determined on the basis of the consensus sequence

	5'-Exon			Intron								3'-Exon
Pre-mRNA	**A/C**	**A**	**G** |	**G**	**T**	**A/G**	**A**	**. . . N**	**C**	**A**	**G** |	**G**
Frequency (%)	70	60	80	100	100	95	70	80	100	100		60

and listed in Table 1. With the exception of the 5' splice site between exon 15 and intron 15, all the exon-intron transitions are in accord with the published (Shapiro and Senapathy, 1987) splice consensus sequence. The sizes of the introns are between 104 bp and 8616 bp. Since only part of intron 6 was isolated, it is not possible to determine the precise length of the hTC gene. Based on the part sequence of ~4660 bp, which was obtained from intron 6, the minimum size of the hTERT gene is 37 kb.

Introns 1-5 and the 5' region of intron 6, are contained in contig 1:

Intron 1: bp 11493-11596 (SEQ ID NO 4);

Intron 2: bp 12951-21566 (SEQ ID NO 5);

Intron 3: bp 21763-23851 (SEQ ID NO 6);

Intron 4: bp 24033-24719 (SEQ ID NO 7);

Intron 5: bp 24900-25393 (SEQ ID NO 8);

5' region of intron 6: bp 25550-26414 (SEQ ID NO 9).

The 3' region of intron 6, and introns 7-15, are located in contig 2 at the following positions:

3' region of intron 6: bp 1-3782 (SEQ ID NO 10);

Intron 7: bp 3879-4858 (SEQ ID NO 11);

Intron 8: bp 4945-7429 (SEQ ID NO 12);

Intron 9: bp 7544-9527 (SEQ ID NO 13);

Intron 10: bp 9600-11470 (SEQ ID NO 14);

Intron 11: bp 11660-15460 (SEQ ID NO 15;

Intron 12: bp 15588-16467 (SEQ ID NO 16);

Intron 13: bp 16530-19715 (SEQ ID NO 17);

Intron 14: 19841-20621 (SEQ ID NO 18);

Intron 15: 20760-21295 (SEQ ID NO 19).

The 3'-untranscribed region is also located in contig 2 at position 21960-25138 (SEQ ID NO 20).

The individual sequences of the abovementioned introns are as follows:

Intron 1 (SEQ ID NO 4)

GTGGGCCTCCCCGGGGTCGGCGTCCGGCTGGGGTTGAGGGCGGCCGGGGGGAACCAGCGACATGCGGAGAGCAGCGCAGG
CGACTCAGGGCGCTTCCCCCGCAG

Intron 2 (SEQ ID NO 5)

GTGAGGAGGTGGTGGCCGTCGAGGGCCCAGGCCCCAGAGCTGAATGCAGTAGGGGCTCAGAAAAGGGGGCAGGCAGAGCC
CTGGTCCTCCTGTCTCCATCGTCACGTGGGCACACGTGGCTTTTCGCTCAGGACGTCGAGTGGACACGGTGATCTCTGCC
TCTGCTCTCCCTCCTGTCCAGTTTGCATAAACTTACGAGGTTCACCTTCACGTTTTGATGGACACGCGGTTTCCAGGCGC
CGAGGCCAGAGCAGTGAACAGAGGAGGCTGGGCGCGGCAGTGGAGCCGGGTTGCCGGCAATGGGGAGAAGTGTCTGGAAG
CACAGACGCTCTGGCGAGGGTGCCTGCAGGTTACCTATAATCCTCTTCGCAATTTCAAGGGTGGGAATGAGAGGTGGGGA
CGAGAACCCCCTCTTCCTGGGGGTGGAGGTAAGGGTTTTGCAGGTGCACGTGGTCAGCCAATATGCAGGTTTGTGTTTA
AGATTTAATTGTGTGTTGACGGCCAGGTGCGGTGGCTCACGCCGGTAATCCCAGCACTTTGGGAAGCTGAGGCAGGTGGA
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TGCAGTGAGCTGAGATTGTGCCATTGTACTCCAGCCTGGGCGACAAGAGTGAAACTCTGTCTTTAAAAAAAAAAAGTGTT
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GTCACGTGTAGGGTGAGTGAGGCGCGGCCCCCGGGTGTCCCTGTCCCGTGCAGCGTGATTGAGGTGTGGCCCCCGGGTGT
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GTCCCTGGGTGTCCCTCCCAGGTATAGGGTGAGTGAGGCACTGTCCCCGGGTGTCCCTGTCACGTGCAGGGTGAGTGAGG
CGCGGCCCCCGGGTGTCCCTCTCAGGTGCAGGGTGAGTGAGGCGCTGTCCCTGGGTGTCCCTGTCTCGTGTAGGGTGAGT
GAGGCTCTGTCCCCAGGTGTCCTTGGCGTTTGCTCACTTGAGCTTGCTCCTGAATGTTTGCTCTTTCTATAGCCACAGCT
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TCTTGGTCACCTCTCCGTTCCATTTTGCTACGGGGACACGGGACTGCAGGCTCTCGCCTCCCGCGTGCCAGGCACTGCAG
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AGACCCTGCTGTGAGCTGGATATGCGGTGTCCGGATGGTGCAGGTCAGGGGTGAGGTCTCCAGGCCCTCGGTGAGCTGGA
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Intron 3 (SEQ ID NO 6)

GTACTGTATCCCCACGCCAGGCCTCTGCTTCTCGAAGTCCTGGAACACCAGCCCGGCCTCAGCATGCGCCTGTCTCCACT
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CAAGGCTCTGACTGCCTGGAGCTCACGTTCTCTTACTTGTAAAATCAGGAGTTTGTGCCAAGTGGTCTCTAGGGTTTGTA
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GCAACCTCCACCTCCTGGGTTTAAGCGATTCACCAGCCTCAGCCTCCTAAGTAGCTGGGATTACAGGCACCTGCCACCAC
GCCTGGCTAATTTTTGTACTTTTAGGAGAGACGGGGTTTCACCATGTTGGCCAGGCTGGTCTCGAACTCATGACCTCAGG
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Intron 4 (SEQ ID NO 7)

GTGGCTGTGCTTTGGTTTAACTTCCTTTTTAAACAGAAGTGCGTTTGAGCCCCACATTTGGTATCAGCTTAGATGAAGGG
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Intron 5 (SEQ ID NO 8)

GTGGGTGCCGGGGACCCCCGTGAGCAGCCCTGCTGGACCTTGGGAGTGGCTGCCTGATTGGCACCTCATGTTGGGTGGAG
GAGGTACTCCTGGGTGGGCCGCAGGGAGTGCAGGTGACCCTGTCACTGTTGAGGACACACCTGGCACCTAGGGTGGAGGC
CTTCAGCCTTTCCTGCAGCACATGGGGCCGACTGTGCACCCTGACTGCCCGGGCTCCTATTCCCAAGGAGGGTCCCACTG
GATTCCAGTTTCCGTCAGAGAAGGAACCGCAACGGCTCAGCCACCAGGCCCCGGTGCCTTGCACCCCAGTCCTGAGCCAG
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CTCCACTCACACAG

5'-region intron 6 (SEQ ID NO 9)

GTAAGGTTCACGTGTGATAGTCGTGTCCAGGATGTGTGTCTCTGGGATATGAATGTGTCTAGAATGCAGTCGTGTCTGTG
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3'-region intron 6 (SEQ ID NO 10)

TGTGGGATTGGTTTTCATGTGTGGGATAGGTGGGGATCTGTGGGATTGGTTTTTATGAGTGGGGTAACACAGAGTTCAAG
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CGGGTTTATAGTAAGTCAGGGGTGTGGAGGCCTCCCCTGGGCTCCCTGTTCTGTTTCTTCCACTCTGGGGTCGTGTGGTG
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CAGAAGAGTTTCACGTGTGCTGATTTCCCGGCTGTTTCCTGCGTAATTGGTGTCTGCTGTTTATCGATGGCCTCCTTCCA
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TCTCAGATCAGCAGTGGCATGCGGTGCTCAGAGGCGCACACACCCTACTGAGAACTGTGCGTGAGAGGGGTCTAGATTCT
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ATGCCCTCTCCTCTGCCGGCAG

Intron 7 (SEQ ID NO 11)

GTCTGGGCACTGCCCTGCAGGGTTGGGCACGGACTCCCAGCAGTGGGTCCTCCCCTGGGCAATCACTGGGCTCATGACCG
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TGAGCTGGGCCATGCCAGGCTGCGACAGCTGCTGCATTCAGGCACCTGCTCACGTTTGACTGCGCGGCCTCTCTCCAGTT
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TGTCCCCCTTCTTAGGAGGACGGGCCGTGTTTGAGCCACGCCCCGCTGAGCGGGCCTCTCAGTGCTGGGTCTGTCCACGT

GGCCCTGTGGCCCTTTGCAGATGTGGTCTGTCCACGTGGCCCTGTGGCTCTTTGCAGATGCCTGTTAGCACTTGCTCGGC
TCTAGGGGACAGTCGTGTCCACCGCATGAGGCTCAGAGACCTCTGGGCGAATTTCCTTGGCTCCCAGGGTGGGGGTGGAG
GTGGCCTGGGCTGCTGGGACCCAGACCCTGTGCCCGGCAGCTGGGCAGCAACTCCTGGATCACATATGCCATCCGGGCCA
CGGTGGGCTGTGTGGGTGTGAGCCCAGCTGGACCCACAGGTGGCCCAGAGGAGACGTTCTGTGTCACACACTCTGCCTAA
GCCCATGTGTGTCTGCAGAGACTCGGCCCGGCCAGCCCACGATGGCCCTGCATTCCAGCCCAGCCCCGCACTTCATCACA
AACACTGACCCCAAAAGGGACGGAGGGTCTTGGCCACGTGGTCCTGCCTGTCTCAGCACCCACCGGCTCACTCCCATGTG
TCTCCCGTCTGCTTTCGCAG

Intron 8 (SEQ ID NO 12)

GTGAGTCAGGTGGCCAGGTGCCATTGCCCTGCGGGTGGCTGGGCGGGCTGGCAGGGCTTCTGCTCACCTCTCTCCTGCCC
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TCCCGAGGCCCCGGAAACATGGCTCGGCTTGCGGCAGCCGGAGCGGAGCAGGTGCCACACGAGGCCTGGAAATGGCAAGC
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GCAGCTTGTCAGCTCCAAGTTACTACTGACGCTGGACACCCGGCTCTCACACGCTTGTATCTCTCTCTCCCGATACAAAA
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AGAATTCGCTGACAAAGTCACCTCCCCAGAGAAGCCACCACGGGCCTCCTTCGTGGTCGTGAATTTTATTAAGATGGATC
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GGTGACTGTGTCTGTCCTGTCCCTAGGACACGGACAGGCCCGAAGCTCTAGTCCCCATCGTGGTCCAGTTTGGCCTCTGA
ATAAAAACGTCTTCAAAACCTGTTGCCCCAAAAACTAAGAACAGAGAGAGTTTCCCATCCCATGTGCTCACAGGGGCGTA
TCTGCTTGCGTTGACTCGCTGGGCTGGCCGGACTCCTAGAGTTGGTGCGTGTGCTTCTGTGCAAAAAGTGCAGTCCTCTT
GCCCATCACTGTGATATCTGCACCAGCAAGGAAAGCCTCTTTTCTTTTCTTTCTTTTTTTTTTTTTGAGACGGAACGTCA
CTGTTGTCTGCCTGGGCTTGAGTGCAGTGGCGCGATCTCAACTCACTGCAACCTCCGCCTCCCGGGTTCCAGCATTTCTC
CTGCCTCAGCCTCCCGAGCAGCTGAGATTACAGGCACCCACCCCCTGCGCCTGGCTAATTTTTGTATTTTTAGTAGAGAG
GGGTTTTTGCCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCAGGTGATCCACCCACCTCGGCCTCCCAAAGTGCTG
GGATTACAGGTGTGAGCCATCACGCCCAGCCGGAAAGCCTCTTTTTAAGGTGACCACCTATAGCGCTTCCCGAAAATAAC
AGGTCTTGTTTTTGCAGTAGGCTGCAAGCGTCTCTTAGCAACAGGAGTGGCGTCCTGTGGGCTCTGGGGATGGCTGAGGG
TCGCGTGGCAGCCATGCCTTCTGTGTGCACCTTTAGGTTCCACGGGGCTATTCTGCTCTCACTGTTTGTCTGAAAACGCA
CCCTTGGCATCCTTGTTTGGAGAGTTTCTGCTTCTCGTTGGTCATGCTGAAACTAGGGGCAAGGTTGTATCCGTTGGCGC
GCAGCGGCTACATGTAGGGTCATGAGTCTTTCACCGTGGACAAATTCCTTGAAAAAAAAAAAAGGAGTCCGGTTAAGCAT
TCATTCCGGGTCAAGTGTCTGGTTCTGTGAATAAACTCTAAGATTTAAGAAACCTTAATGAAAGAAAACCTTGATGATTC
AGAGCAAGGATGTGGTCACACCTGTGGCTGGATCTGTTTCAGCCGCCCCAGTGCATGGTGAGAGTGGGGAGCAGGGATTG
TTTGTTCAGAGGTCTCATCTGGTATGTTTCTGAGGTGTTTGCCGGCTGAATGGTAGACGTGTCGTTTGTGTGTATGAGGT
TCTGTGTCTGTGTGTGGCTCGGTTTGAGTGTACGCATGTCCAGCACATGCCCTGCCCGTCTCTCACCTGTGTCTTCCCGC
CCCAG

Intron 9 (SEQ ID NO 13)

GTGAGGCCTCCTCTTCCCCAGGGGGGCTTGGGTGGGGGTTGATTTGCTTTTGATGCATTCAGTGTTAATATTCCTGGTGC
TCTGGAGACCATGACTGCTCTGTCTTGAGGAACCAGACAAGGTTGCAGCCCCTTCTTGGTATGAAGCCGCACGGGAGGGG

TTGCACAGCCTGAGGACTGCGGGCTCCACGCAGGCTCTGTCCAGCGGCCATGTCCAGAGGCCTCAGGGCTCAGCAGGCGG
GAGGGCCGCTGCCCTGCATGATGAGCATGTGAATTCAACACCGAGGAAGCACACCAGCTTCTGTCACGTCACCCAGGTTC
CGTTAGGGTCCTTGGGGAGATGGGGCTGGTGCAGCCTGAGGCCCCACATCTCCCAGCAGGCCCTCGACAGGTGGCCTGGA
CTGGGCGCCTCTTCAGCCCATTGCCCATCCCACTTGCATGGGGTCTACACCCAAGGACGCACACACCTAAATATCGTGCC
AACCTAATGTGGTTCAACTCAGCTGGCTTTTATTGACAGCAGTTACTTTTTTTTTTTTAATACTTTAAGTTCTAGGGTAC
ATGTGCACGACGTGCAGGTTAGTTACATATGTATACATGTGCCATGTTGGTGTGCTGCACCCATTAACTCATCATTTACA
TTAGGTATATCTCCTAATGCTATCCCTCCCCACTCCCCCCATCCCATGACAGGCCCTGGTGTGTGATGTTCCCCACCCTG
TGTCCAAGTGTTCTCATTGTTCAGTTCCCACCTGTGAGTGAGAACATGTGGTGTTTGGTTTTCTTTCCTTGCAATAGTTT
GCTCAGAGTGATGGTTTCCAGCTTCGTCCATGTCCCTACAAAGGACATGAACTCATCCTTTTTTATGACTGCATAGTATT
CCGTGGTGTATATGTGCCACATTTTCTTAATCCAGTCTATCATCGATGGACATTTGGGTTGGTTGCAAGTCTTTGCTACT
GTGAATAGTGCCGCAATAAACATACGTGTGCATGTGTCTTTATAGCAGCATGATTTATAATCCTTTGGGTATATACCCAG
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CTAGTTTACACTCCCACCAACAGTGTAAAAGTGTTCTGGTGCTGGAGAGGATGTGGACAGCAGTTATTTTTTTATGAAAA
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TCCGGGTTTTTCCTGTGCATCTTTTGAAACTCTAGCTCCAATTATAGCATGTACAGTGGATCAAGGTTCTTCTTCATTAA
GGTTCAAGTTCTAGATTGAAATAAGTTTATGTAACAGAAACAAAAATTTCTTGTACACACAACTTGCTCTGGGATTTGGA
GGAAAGTGTCCTCGAGCTGGCGGCACACTGGTCAGCCCTCTGGGACAGGATACCTCTGGCCCATGGTCATGGGGCGCTGG
GCTTGGGCCTGAGGGTCACACAGTGCACCATGCCCAGCTTCCTGTGGATAGGATCTGGGTCTCGGATCATGCTGAGGACC
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GCTGATGGTAAACACTGAGTACTTATAATGAATGAGGAATTGCTGTAGCAGTTAACTGTAGAGAGCTCGTCTGTTGGAAA
GAAATTTAAGTTTTTCATTTAACCGCTTTGGAGAATGTTACTTTATTTATGGCTGTGTAAATTGTTTGACATTCAGTCCC
TCGTAGACAGATACTACGTAAAAAGTGTAAAGTTAACCTTGCTGTGTATTTTCCCTTATTTTAG

Intron 10 (SEQ ID NO 14)

GTGAGGCCCGTGCCGTGTGTCTGTGGGGACCTCCACAGCCTGTGGGCTTTGCAGTTGAGCCCCCCGTGTCCTGCCCCTGG
CACCGCAGCGTTGTCTCTGCCAAGTCCTCTCTCTCTGCCGGTGCTGGATCCGCAAGAGCAGAGGCGCTTGGCCGTGCACC
CAGGCCTGGGGGCGCAGGGGCACCTTCGGGAGGGAGTGGGTACCGTGCAGGCCCTGGTCCTGCAGAGACGCACCCAGGTT
ACACACGTGGTGAGTGCAGGCGGTGACCTGGCTCCTGCTGCTCTTTGGAAAGTCAAGAGTGGCGGCTCCTGGGGCCCCAG
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GAGCAGGAGCTGCTGAGTGAGCTGGCCCACAGCGTTCGCTGCGGTCACGTTCCTGCGTGGGGTTGTTTGGGATCGGTGGG
AGAATTTGGATTTGCTGAGTGCTGCTGTCTTGAACCACGGAGATGGCTAGGAGTGGGTTTCAGAGTTGATTTTTGTGAAT
CAAACTAAAATCAGGCACAGGGGACCTGGCCTCAGCACAGGGGATTGTCCAATGTGGTCCCCCTCAAGGGCGCCCCACAG
AGCCGGTGGGCTTGTTTTAAAGTGCGATTTGACGAGGGACGAGAAACCTTGAAAGCTGTAAAGGGAACCCTCAGAAAATG
TGGCCGCCAGGGGTGGTTTCAGGTGCTTTGCTGGGCTGTGTTTGTGAAAACCCATTTGGACCCGCCCTCCAAGTCCACCC
TCCAGGTCCACCCTCCAGGGCCGCCCTGGGCTGGGGGTATGCCTGGCGTTCCTTGTGCCGCAGCCCGGAGCACAGCAGGC
TGTGCACATTTAAATCCACTAAGATTCACTCGGGGGGAGCCCAGGTCCCAAGCAACTGAGGGCTCAGGAGTCCTGAGGCT
GCTGAGGGGACAGAGCAGACGGGGAACGCTGCTTCTGTGTGGCAAGTTCCTGAGGGTGCTGGCCAGGGAGGTGGCTCAGA
GTGTATGTTGGGGTCCCACCGGGGGCAGAACTCTGTCTCTGATGAGTCGGCAGCCATGTAACAGGAAGGGGTGGCCACAG
GGAGCTGGGAATGCACCAGGGGAGCTGCGCAGCTGGCCGAGGTCCCAGGGCCAGGCCACAGGAAGGGCAGGGGGACGCCC
GGGGCCACAGCAGAGGCCGCAGGAAGGGAAGGGGATGCCCAGGCCAGAGCAGAGGCTACCGGGCACAGGGGGGCTCCCTG
AGCTGGGTGAGCGAGGCTCATGACTCGGCGAGGGAACCTCCTTGACGTGAAGCTGACGACTGGTGTTGCCCAGCTCACAG
CCCAGCCAGGTCCCGCGCCTGAGCAGGAACTCAGAACCCTCCCCTTTGTCTAAAGCACAGCAGATGCCTTCAGGGCATCT
AGGAGAAAACAGGCAAAGTCGTTGAGAAACGTCTTAAAAGAAGGTGGGATGGTGGCAATTTCTTGTCCAGATTTTAGTCT
GCCCCGGACCACAGATGAGTCTATAACGGGATTGTGGTGTTGCCATGGGGACACATGAGATGGACCATCACAGAGGCCAC
TGGGGCTGCACCTCCCATCTGAGTCCTGGCTGTCCCGGGTCCAGGCCAGGTTCTTGCATGCTCACCTACCTGTCCTGCCC

GGGAGACAGGGAAAGCACCCCGAAGTCTGGAGCAGGGCTGGGTCCAGGCTCCTCAGAGCTCCTGCCAGGCCCAGCACCCT
GCTCCAAATCACCACTTCTCTGGGGTTTTCCAAAGCATTTAACAAGGGTGTCAGGTTACCTCCTGGGTGACGGCCCCGCA
TCCTGGGGCTGACATTGCCCCTCTGCCTTAG

Intron 11 (SEQ ID NO 15)

GTGAGCGCACCTGGCCGGAAGTGGAGCCTGTGCCCGGCTGGGGCAGGTGCTGCTGCAGGGCCGTTGCGTCCACCTCTGCT
TCCGTGTGGGGCAGGCGACTGCCAATCCCAAAGGGTCAGAGGCCACAGGGTGCCCCTCGTCCCATCTGGGGCTGAGCAGA
AATGCATCTTTCTGTGGGAGTGAGGGTGCTCACAACGGGAGCAGTTTTCTGTGCTATTTTGGTAAAAGGAAATGGTGCAC
CAGACCTGGGTGCACTGAGGTGTCTTCAGAAAGCAGTCTGGATCCGAACCCAAGACGCCCGGGCCCTGCTGGGCGTGAGT
CTCTCAAACCCGAACACAGGGGCCCTGCTGGGCATGAGTCCCTCTGAACCCGAGACCCTGGGGCCCTGCTGGGCGTGAGT
CTCTCCGAACCCAGAGACTTCAGGGCCCTTTTGGGCGTGAGTCTCTCCGCTGTGAGCCCCACACTCCAAGGCTCATCCAC
AGTCTACAGGATGCCATGAGTTCATGATCACGTGTGACCCATCAGGGGACAGGGCCATGGTGTGGGGGGGTCTCTACAA
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ACAGAGTGAGACTTCATCTTAAAAAAAAAAAAAAAAAGTATCAGCATTCCAAAACCATAGTGGACAGGTGTTTTTTTATTC
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CAGAAACGTGTGTTAATGTGGTATGTGGCACAGCTGATGGAAAAGAGAGTGTGTGTGTAATTTTTTTTTCTGAGAAAACT
GACTGGAAGCAAATAAGTTGTGTCTTTACAGCATATACCAGAGCAGATTCTAGGTAGAAGAGGAGACACATGCAAACAAC
ACCAGCAACAGAAATAAAACAAAAGACTCAAAGGGAAGGGAGGTGAACGTTCCCTGGTTTGGTGTTGGGGAAGGACACAC
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AGGGTGAGCCGGAGCCCAAGGTCGTGTTGGGGATGGCTGTGAAAGAAGAAATGGACGTCTGATGCACACTTGGGAAGGTC
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GAGACCTGTCCCCATCCCTCATGCTGGCTCCTTTTCTGGGCTTGCCAAGAGCCAGCATCAGGTTGAGGCAAGCTGGAAAG
ACTTTTCTGGAAAGCAGCTTGTTTGCATGGAAGTCCTCACAATGTCCTGTGTCTTCCCAGTAATTCCACTTCTGAAGTGA
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CAAATACAGGGCTAAGGAGATATTATGCATCACAAAACTTGCTCTGCCATTAAACATTTTTCAAAGAATTTTTGAAGAAT
GTTTAATGGCACAAAACGTTTATTTCAATGTAGCAGTGTTCAAAGCTGGATGTAAAAGAACACACCCCAGGAGCCTGCCG
TGAATGTCATGTGTGTTCATCTTTGGACATGGACATACATGGGCAGTGAGTGGTGGTGAGGCCCTGGAGGACATCGGTGG

GATGCCTCCATCCTGCCCCTCTGGAGACACCATGTGTGCCACGTGCACTCACTGGAGCCCTGTTTAGCTGGTGCCACCTG
GCTCTTCCATCCCTGAGATTCAAACACAGTGAGATTCCCCACGCCCAACTCAGTGTTCTCCCACAAAAAACCTGAGTCAC
ACCTGTGTTCACTCGAGGGACGCCCGGGAGCCAGGGCTCCACAGTTTATTATGTGTTTTTGGCTGAGTTATGTGCAGATC
TCATCAGGGCAGATGATGAGTGCACAAACACGGCCGTGCGAGGTTTGGATACACTCAACATCACTAGCCAGGTCCTGGTG
GAGTTTGGTCATGCAGAGTCTGGATGGCATGTAGCATTTGGAGTCCATGGAGTGAGCACCCAGCCCCCTCGGGCTGCAGC
GCATGCCCCAGGCAGGACAAGGAAGCGGAGGAAGGCAGGAGGCTCTTTGGAGCAAGCTTTGCAGGAGGGGGCTGGGTGT
GGGGCAGGCACCTGTGTCTGACATTCCCCCCTGTGTCTCAG

Intron 12 (SEQ ID NO 16)

GTGAGCAGGCTGATGGTCAGCACAGAGTTCAGAGTTCAGGAGGTGTGTGCGCAAGTATGTGTGTGTGTGTGTGCGCGCGT
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TGCATGTGTGTTCGTGCACAGTCGTGTGGGCATTCACGTGAGGTGCATGCGTGTGGGTGTGCAGTGTGAGTAGCATGTGT
GCACATAACATGTATTGAGGGGTCCTCGTGTTCACCCCGCTAGGTCCTCAGCACCAGTGCCACTCCTTACAGGATGAGAC
GGGGTCCCAGGCCTTGGTGGGCTGAGGCTCTGAAGCTGCAGCCCTGAGGGCATTGTCCCATCTGGGCATCCGCGTCCACT
CCCTCTCCTGTGGGCTTCTGTGTCCACTCCCCCTCTCCTGTGGGCATTTACATCCACTCCACTCCCTCTCTCCTGTGGGC
ATCCGCGTCCACTCCCCCTCTCTGTGGGCATCTGCGTCCACCTCCCCTCTCTGTGGGCATTTGCGTCCACTCCCTCTCCT
GGTTCCTTCCTGTCTTGGCCGAGCCTCGGGGGCAGGCAGATGACACAGAGTCTTGACTCGCCCAGGGTGGTTCGCAGCTG
CCGGGTGAGGGCCAGGCCGGATTTCACTGGGAAGAGGGATAGTTTCTTGTCAAAATGTTCCTCTTTCTTGTTCCATCTGA
ATGGATGATAAAGCAAAAAGTAAAAACTTAAAATCCCAGAGAGGTTTCTACCGTTTCTCACTCTTTCTTGGCGACTCTAG

Intron 13 (SEQ ID NO 17)

GTGAGCCGCCACCAAGGGGTGCAGGCCCAGCCTCCAGGGACCCTCCGCGCTCTGCTCACCTCTGACCCGGGGCTTCACCT
TGGAACTCCTGGGTTTTAGGGGCAAGGAATGTCTTACGTTTTCAGTGGTGCTGCTGCCTGTGCACAGTTCTGTTCGCGTG
GCTCTGTGCAAAGCACCTGTTCTCCATCTCTGGGTAGTGGTAGGAGCCGGTGTGGCCCCAGGTGTCCCCACTGTGCCTGT
GCACTGGCCGTGGGACGTCATGGAGGCCATCCCAGGGCAGCAGGGGCATGGGGTAAAGAGATGTTTATGGGGAGTCTTAG
CAGAGGAGGCTGGGAAGGTGTCTGAACAGTAGATGGGAGATCAGATGCCCGGAGGATTTGGGGTCTCAGCAAAGAGGGCC
GAGGTGGGTGCAGGTGAGGGTCGCTGGCCCCACCCCCGGGAAGGTGCAGCAGAGCTGTGGCTCCCCACACAGCCCGGCCA
GCACCTGTGCTCTGGGCATGGCTGTGCTCCTGGAACGTTCCCTGTCCTGGCTGGTCAGGGGGTGCCCCTGCCAAGAATCG
ACAACTTTATCACAGAGGGAAGGGCCAATCTGTGGAGGCCACAGGGCCAGCTTCTGCCTGGAGTCAGGGCAGGTGGTGGC
ACAAGCCTCGGGGCTGTACCAAAGGGCAGTCGGGCACCACAGGCCCGGGCCTCCACCTCAACAGGCCTCCCGAGCCACTG
GGAGCTGAATGCCAGGAGGCCGAAGCCCTCGCCCCATGAGGGCTGAGAAGGAGTGTGAGCATTTGTGTTACCCAGGGCCG
AGGCTGCGCGAATTACCGTGCACACTTGATGTGAAATGAGGTCGTCGTCTATCGTGGAAACCCAGCAAGGGCTCACGGGA
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CCCTCGTGCAAGCTGCTTGACTCCTTTCCGGAAACCCTTGGGGTGTGCTGGATACAGGTGCCACTGAGGACTGGAGGTGT
CTGACACTGTGGTTGACCCCAGGGTCCAGCTGGCGTGCTTGGGGCCTCCTTGGGCCATGATGAGGTCAGAGGAGTTTTCC
CAGGTGAAAACTCCTGGGAAACTCCCAGGGCCATGTGACCTGCCACCTGCTCCTCCCATATTCAGCTCAGTCTTGTCCTC
ATTTCCCCACCAGGGTCTCTAGCTCCGAGGAGCTCCCGTAGAGGGCCTGGGCTCAGGGCAGGGCGGCTGAGTTTCCCCAC
CCATGTGGGGACCCTTGGGTAGTCGCTTGATTGGGTAGCCCTGAGGAGGCCGAGATGCGATGGGCCACGGGCCGTTTCCA
AACACAGAGTCAGGCACGTGGAAGGCCCAGGAATCCCCTTCCCTCGAGGCAGGAGTGGGAGAACGGAGAGCTGGGCCCCG
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TATTTCTCCCTTTGGAAGAGAGCCCCTCACCCATGCTAGGTGTTTCCCTCCTGGGTCAGGAGCGTGGCCGTGTGGCAACC

CCGGGACCTTAGGCTTATTTATTTGTTTAAAAACATTCTGGGCCTGGCTTCCGTTGTTGCTAAATGGGAAAAGACATCC
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CAGGAAGTCAGTGAGACCAGGTACATGGGGGGCTCAGGCAGTGGGTGAGATGAGGTACACGGGGGGCTCAGGCAGTGGGT
GAGGCCAGGTACATGGGGGGCTCAGGCACTGGGTGAGATGAGGTACACGGGGGGCTCAGGCAGAGGGTCAGACCAGGTAC
ACGGGGGCTCTGATCACACGCACATATGAGCACATGTGCACATGTGCTGTTTCATGGTAGCCAGGTCTGTGCACACCTGC
CCCAAAGTCCCAGGAAGCTGAGAGGCCAAAGATGGAGGCTGACAGGGCTGGCGCGGTGGCTCACACCTGTAGTCCCAGCA
CTTTGGGAGGCCGAGGCGAGAGGATCCCTTGAGCCCAGGAGTTTAAGACCAGCCTGAGCAACATAGTAGAACCCCATCTC
TATGAAAAATAAAAACAAAAATTAGCTGAACATGGTGGTGTGCGCCTGTAGTTCCAATACTTGGGAGGCTGAAGTGGGAG
GATCACTTGAGCCCAGGAGGTGGAAGCTGCAGTGAGCTGAGATTGCACCACTGTACTGCAGCCTGGGTGACAGAGTGAGA
GCCCATCTCAACAACAACAAAGAAGACTGACAAATGCAGTTTCTTGGAAAGAAACATTTAGTAGGAACTTAACCTACACA
CAGAAGCCAAGTCGGTGTCTCGGTGTCAGTGAGATGAGATGATGGGTCCTCACACCATCACCCCAGACCCAGGGTTTATG
CACCACAGGGGCGGGTGGCTCAGAAGGGATGCGCAGGACGTTGATATACGATGACATCAAGGTTGTCTGACGAAGGGCAG
GATTCATGATAAGTACCTGCTGGTACACAAGGAACAATGGATAAACTGGAAACCTTAGAGGCCTTCCCGGAACAGGGGCT
AATCAGAAGCCAGCATGGGGGGCTGGCATCCAGGATGGAGCTGCTTCAGCCTCCACATGCGTGTTCATACAGATGGTGCA
CAGAAACGCAGTGTACCTGTGCACACACAGACACGCAGCTACTCGCACACACAAGCACACACACAGACATGCATGCATGC
ATCCGTGTGTGTGCACCTGTGCCCATGAGGAAACCCATGCATGTGCATTCATGCACGCACACAGGCACCGGTGGGCCCAT
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Intron 14 (WEQ ID NO 18)

GTATGTGCAGGTGCCTGGCCTCAGTGGCAGCAGTGCCTGCCTGCTGGTGTTAGTGTGTCAGGAGACTGAGTGAATCTGGG
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GGGGTGAGCAGAGCACCTGATGGAAGGGACAGGAGCTGTCTGGGAGCTGCCATCCTTCCCACCTTGCTCTGCCTGGGGAA
GCGCTGGGGGGCCTGGTCTCTCCTGTTTGCCCCATGGTGGATTTGGGGGGCCTGGCCTCTCCTGTTTGCCCTGTGGTGG
GATTGGGCTGTCTCCCGTCCATGGCACTTAGGGCCCTTGTGCAAACCCAGGCCAAGGGCTTAGGAGGAGGCCAGGCCCAG
GCTACCCCACCCCTCTCAGGAGCAGAGGCCGCGTATCACCACGACAGAGCCCCGCGCCGTCCTCTGCTTCCCAGTCACCG
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CCTGAGCTTAACAGCTTCTACTTTCTGTTCTTTCTGTGTTGTGGAAATTTCACCTGGAGAAGCCGAAGAAAACATTTCTG
TCGTGACTCCTGCGGTGCTTGGGTCGGGACAGCCAGAGATGGAGCCACCCCGCAGACCGTCGGGTGTGGGCAGCTTTCCG
GTGTCTCCTGGGAGGGGAGCTGGGCTGGGCCTGTGACTCCTCAGCCTCTGTTTTCCCCCAG

Intron 15 (SEQ ID NO 19)

GCAAGTGTGGGTGGAGGCCAGTGCGGGCCCCACCTGCCCAGGGGTCATCCTTGAACGCCCTGTGTGGGGCGAGCAGCCTC
AGATGCTGCTGAAGTGCAGACGCCCCCGGGCCTGACCCTGGGGGCCTGGAGCCACGCTGGCAGCCCTATGTGATTAAACG
CTGGTGTCCCCAGGCCACGGAGCCTGGCAGGGTCCCCAACTTCTTGAACCCCTGCTTCCCATCTCAGGGGCGATGGCTCC
CCACGCTTGGGAGCCTTCTGACCCCTGACCTGTGTCCTCTCACAGCCTCTTCCCTGGCTGCTGCCCTGAGCTCCTGGGGT
CCTGAGCAAGTTCTCTCCCCGCCCCGCCGCTCCAGCGTCACTGGGCTGCCTGTCTGCTCGCCCCGGTGGAGGGGTGTCTG
TCCCTTCACTGAGGTTCCCACCAGCCAGGGCCACGAGGTGCAGGCCCTGCCTGCCCGGCCACCCACACGTCCTAGGAGGG
TTGGAGGATGCCACCTCTGGCCTCTTCTGGAACGGAGTCTGATTTTGGCCCCGCAG

3'-untranscribed region (SEQ ID NO 20)

ATCTCATGTTTGAATCCTAATGTGCACTGCATAGACACCACTGTATGCAATTACAGAAGCCTGTGAGTGAACGGGGTGGT
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GCCTGGCTGGGCCTGGGAGGTTTCTGATGCTGTGAGGCAGGAGGGGAAGGAGGGTAGGGGATAGACAGTGGGAGCCCCCA
CCCTGGAAGACATAACAGTAAGTCCAGGCCCGAAGGGCAGCAGGGATGCTGGGGGCCCAGCTTGGGCGGCGGGGATGATG
GAGGGCCTGGCCAGGGTGGCAGGGATGATGGGGGCCCCAGCTGGGGTGGCAGGGGTGATGGGGGGGCTGGTCTGGGTGG

CGGGGAAGATGGGGAAGCCTGGCTGGGCCCCCTCCTCCCCTGCCTCCCACCTGCAGCCGTGGATCCGGATGTGCTTCCCT
GGTGCACATCCTCTGGGCCATCAGCTTTCATGGAGGTGGGGGGCAGGGGCATGACACCATCCTGTATAAAATCCAGGATT
CCTCCTCCTGAACGCCCCAACTCAGGTTGAAAGTCACATTCCGCCTCTGGCCATTCTCTTAAGAGTAGACCAGGATTCTG
ATCTCTGAAGGGTGGGTAGGGTGGGGCAGTGGAGGGTGTGGACACAGGAGGCTTCAGGGTGGGGCTGGTGATGCTCTCTC
ATCCTCTTATCATCTCCCAGTCTCATCTCTCATCCTCTTATCATCTCCCAGTCTCATCTGTCTTCCTCTTATCTCCCAGT
CTCATCTGTCATCCTCTTACCATCTCCCAGTCTCATCTCTTATCCTCTTATCTCCTAGTCTCATCCAGACTTACCTCCCA
GGGCGGGTGCCAGGCTCGCAGTGGAGCTGGACATACGTCCTTCCTCAGGCAGAAGGAACTGGAAGGATTGCAGAGAACAG
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AGGGCCCTGCGTGAGTGGCTCCAGAGCCTTCCAGCAGGTCCCTGGTGGGGCCTTATGGTATGGCCGGGTCCTACTGAGTG
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GCGTCATTTATTGCTGCTGCTTCAGAGAATGTCTGAGTGACCGAGCCTAATGTGTATGGTGGGCCCAAGTCCACAGACTG
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Characterization of the exons showed, interestingly, that the functionally important hTC protein domains which are described in our Patent Application PCT/EP/98/03469 are arranged on separate exons. The telomerase-characteristic T motif is located on exon 3. The RT (reverse transcriptase) motifs 1-7, which are important for the catalytic function of the telomerase, are located on the following exons: RT motifs 1 and 2 on exon 4, RT motif 4 on exon 9, RT motif 5 on exon 10, and RT motifs 6 and 7 on exon 11. RT motif 3 is shared by exons 5 and 6 (see Fig. 8).

Elucidation of the exon-intron structure of the hTC gene also shows that the four deletions or insertion variants of the hTC cDNA which were described in our Patent Application PCT/EP/98/03469, as well as three additional hTC insertion variants which are described in the literature (Kilian et al., 1997), in all probability represent alternative splicing products. As shown in Fig. 8, the splicing variants can be divided into two groups: deletion variants and insertion variants.

The hTC variants in the deletion group lack specific sequence segments. The 36 bp in-frame deletion in variant DEL1 in all probability results from using an alternative 3' splice acceptor sequence in exon 6, resulting in a part of RT motif 3 being lost. In variant DEL2, the normal 5' splice donor and 3' splice acceptor sequences of introns 6, 7 and 8 are not used. Instead exon 6 is fused directly to exon 9, resulting in a displacement arising in the open reading frame and a stop codon appearing in exon 10. Variant Del3 is a combination of variants 1 and 2.

The insertion variant group is characterized by the insertion of intron sequences which lead to premature cessation of translation. Instead of the 5' splice donor sequence of intron 5, which is normally used, use is made, in variant INS1, of an alternative, 3'-located splice site, resulting in the insertion of the first 38 bp from intron 4 between exon 4 and exon 5. The insertion, in variant INS2, of a region of the intron 11 sequence likewise results from using an alternative 5' splice donor sequence in intron 11. Since this variant was only described inadequately in the

literature (Kilian et al., 1997), it is not possible to determine the precise alternative 5' splice donor sequence in this variant. The insertion of intron 14 sequences between exon 14 and exon 15 in variant INS3 comes from using an alternative 3' splice acceptor sequence, resulting in the 3' part of intron 14 not being spliced.

The hTC variant INS4 (variante 4), which is described in our Patent Application PCT/EP/98/03469, is characterized by exon 15, and the 5' part region of exon 16, being replaced by the first 600 bp of intron 14. This variant can be attributed to the use of an alternative internal 5' splice donor sequence in intron 14 and an alternative 3' splice acceptor sequence in exon 16, resulting in an altered C terminus.

The *in vivo* generation of hTC protein variants which are probably non-functional and which could interfere with the function of the complete hTC protein constitutes a possible mechanism, in addition to transcription regulation, for controlling hTC protein function. The function of the hTC splicing variants is not yet known. Although most of these variants presumably encode proteins without reverse transcriptase activity, they could nevertheless play a crucial role as transdominant-negative telomerase regulators by, for example, competing for interaction with important binding partners.

The search for possible transcription factor binding sites was carried out using the „find pattern" algorithm from the Genetics Computer Group (Madison, USA) GCG Sequence Analysis program package. This resulted in the identification of a variety of potential binding sites for transcription factors in the nucleotide sequence of intron 2, which binding sites are listed in Tab. 2. In addition, an Sp1 binding site was found in intron 1 (pos. 43), and a c-Myc binding site was found in the 5'-untranslated region (cDNA position 29-34, cf. Fig. 6).

Example 6

In order to ascertain the start point(s) of hTC transcription in HL 60 cells, the 5' end of the hTC mRNA was determined by means of primer extension analysis.

2 µg of polyA$^+$ RNA from HL-60 cells were denaturated at 65°C for 10 min. 1 µl of RNasin (30-40 U/ml) and 0.3-1 pmol of radioactively labelled primer (5'GTTAAGTTGTAGCTTACACTGGTTCTC 3'; 2.5-8x10^5 cpm) were added for primer annealing, and the whole was incubated, at 37°C for 30 min, in a total volume of 20 µl. After the addition of 10 µl of 5xreverse transcriptase buffer (from Gibco-BRL), 2 µl of 10 mM dNTPs, 2 µl RNasin (see above), 5 µl of 0.1 M DTT (from Gibco-BRL) 2 µl of ThermoScript RT (15 U/µl; from Gibco-BRL) and 9 µl of DEPC-treated water, primer extension took place, at 58°C for 1 h, in a total volume [lacuna]. The reaction was stopped by adding 4 µl of 0.5 M EDTA, pH 8.0, and the RNA was degraded, at 37°C for 30 min, after having added 1 µl of RNaseA (10 mg/ml). 2.5 µg of sheared calf thymus DNA and 100 µl of TE were then added, and the mixture was extracted once with 150 µl of phenol/chloroform (1:1). The DNA was precipitated, at -70°C for 45 min, after adding 15 µl of 3 M Na acetate and 450 µl of ethanol, and then centrifuged at 14,000 rpm for 15 min. The precipitate was washed once with 70% ethanol, dried in air and dissolved in 8 µl of sequencing stop solution. After 5 min of denaturation at 80°C, the samples were loaded onto a 6% polyacrylamide gel and fractionated electrophoretically (Ausubel et al., 1987) (Fig. 5).

In this connection, a main transcription start site was identified which is located 1767 bp 5' of the ATG start codon of the hTC cDNA sequence (nucleotide position 3346 in Fig. 4). In addition to this, the nucleotide sequence around this main transcription start (TTA$_{+1}$TTGT) represents an initiator element (Inr), which, in 6 out of 7 nucleotides, matches the consensus motif (PyPyA$_{+1}$Na/tPyPy) (Smale, 1997) of an initiator element.

It was not possible to identify any unambiguous TATA box in the immediate vicinity of the experimentally identified main transcription start, which means that the hTC promoter has probably to be classified in the family of TATA-less promoters (Smale, 1997). However, a potential TATA box from nucleotide position 1306 to nucleotide position 1311 (Fig. 4) was found by means of bioinformatics analysis. The subsidiary transcription starts which were additionally observed around the main transcription start have also been described in the case of other TATA-less promoters (Geng and Johnson, 1993), for example in the strongly regulated promoters of some cell cycle genes (Wick *et al.*, 1995).

Example 7

In addition to the start point of the hTC transcript which was described in Example 6 and identified in HL60 cells, a further transcription start region was also identified in HL60 cells. With the aid of RT-PCR analyses, the region of the hTC gene transcription start in HL60 cells was localized to bp –60 to bp –105.

The cDNA for this was synthesized using a First Strand cDNA Synthesis kit (Clontech), in accordance with the manufacturer's instructions, and employing 0.4 µg of HL60 cell polyA RNA (Clontech) and the gene-specific primer GSP13 (5'-CCTCCAAAGAGGTGGCTTCTTCGGC-3', cDNA position 920-897). In a final volume of 50 µl, 10 pmol dNTP mix were added to 1 µl of cDNA, and a PCR reaction was carried out in 1xPCR reaction buffer F (PCR-Optimizer kit from InVitrogen) and using one unit of platinum Taq DNA polymerase (from Gibco/BRL). 10 pmol of each of the 5' and 3' primers defined below were added as primers. The PCR was carried out in 3 steps. A two-minute denaturation at 94°C was followed by 36 PCR cycles in which the DNA was first of all denatured at 94°C for 45 sec and, after that, the primers were annealed, and the DNA chain was extended at 68°C for 5 min. The cycles were concluded by a chain extension at 68°C for 10 min. In all, six different 5' PCR primers (primer HTRT5B: 5'-CGCAGCCACTACCGCGAGGTGC-3', cDNA position 105 to 126; primer C5S:

5'-CTGCGTCCTGCTGCGCACGTGGGAAGC-3', 5'-flanking region -49 to -23; primer PRO-TEST1: 5'-CTCGCGGCGCGAGTTTCAGGCAG-3', 5'-flanking region –74 to -52; primer PRO-TEST2: 5'-CCAGCCCCTCCCCTTCCTTTCC-3', 5'-flanking region –112 to -91; primer PRO-TEST4: 5'-CCAGCTCCGCCTCCTCCGCGC-3', 5'-flanking region –191 to -171; primer RP-3A: 5'-CTAGGCCGATTCGACCTCTCTCC-3', 5'-flanking region –427 to -405) were combined with the 3' PCR primer C5Rback (5'-GTCCCAGGGCACGCACACCAG-3', cDNA position 245 to 225). Genomic DNA was also employed for the PCR, as a control, in addition to the Oligo dT- and GSP13-primed cDNAs. As Fig. 9 shows, a PCR product was only obtained with the primer combinations HTRT5B-C5Rback, C5S-C5Rback and PRO-TEST1-C5Rback, indicating that the start point for hTC transcription lies in the region between bp-60 and bp-105.

Example 8

Several extremely GC-rich regions, so-called CpG Islands, are located in the isolated 5'-flanking region, of about 11.2 kb in size, of the hTC gene. One CpG Island, having a GC content of > 70%, extends from bp – 1214 into intron 2. Two further GC-rich regions having a GC content of > 60% extend from bp –3872 to bp –3113 and from bp –5363 to bp –3941, respectively. The positions of the CpG Islands are shown graphically in Fig. 11.

The search for possible transcription factor binding sites was carried out using the "Find Pattern" algorithm from the Genetics Computer Group (Madison, USA) GCG Sequence Analysis program package. This resulted in the identification of a variety of potential binding sites in the region up to -900 bp upstream of the translation start codon ATG: five Sp1 binding sites, one c-Myc binding site, and one CCAC box (Fig. 10). In addition, a CCAAT box and a second c-Myc binding site were found at positions –1788 and –3995, respectively, of the 5'-flanking region.

Example 9

In order to analyse the activity of the hTC promoter, PCR amplification was used to generate four hTC promoter sequence segments of differing length, which segments were cloned into the Promega vector pGL2 5' in front of the luciferase reporter gene. The 8.5 kb SacI fragment which was subcloned from phage clone P12 was selected as the DNA source for the PCR amplification. In a final volume of 50 µl, 10 pmol of dNTP mix were added to 35 ng of this DNA, and a PCR reaction was carried out in 1xPCR reaction buffer (PCR-Optimizer kit from InVitrogen) and using one unit of platinum Taq DNA polymerase (from Gibco/BRL). In each case 20 pmol of the 5' and 3' primers which are defined below were added as primers. The PCR was carried out in three steps. A two-minute denaturation at 94°C was followeed by 30 PCR cycles in which the DNA was first of all denaturated at 94°C for 45 sec, after which the primers were annealed, and the DNA chain was extended, at 68°C for 5 min. The cycles were concluded by a chain extension at 68°C for 10 min. The selected 3' PCR primer was in each case the primer PK-3A (5'-GCAAGCTTGACGCAGCGCTGCCTGAAACTCG-3', position –43 to -65), which primer recognizes a sequence region 42 bp upstream of the ATG START codon. A promoter fragment of 4051 bp in size (NPK8) was amplified by combining the PK-3A primers with the 5' PCR primer PK-5B (5'-CCAGATCTCTGGAACACAGAGTGGCAGTTTCC-3', position –4093 to -4070). Combining the pair of primers PK-3A and PK-5C (5'-CCAGATCTGCATGAAGTGTGTGGGGATTTGCAG-3', position –3120 to -3096) led to the amplification of a promoter fragment of 3078 bp in size (NPK15). Use of the primer combination PK-3A and PK-5D (5'-GGAGATCTGATCTTGGCTTACTGCAGCCTCTG-3', position –2110 to -2087) amplified a promoter fragment of 2068 bp in size (NPK22). Finally, using the primer combination PK-3A and PK-5E (5'-GGAGATCTGTCTGGATTCCTGGGAAGTCCTCA-3', position –1125 to -1102) led to the amplification of a promoter fragment of 1083 bp in size (NPK27).

The PK-3A primer contains a HindIII recognition sequence. The different 5' primers contain a BglII recognition sequence.

The resulting PCR products were purified using the Qiagen QIA quick spin PCR purification kit, in accordance with the manufacturer's instructions, and then digested with the restriction enzymes BglII and HindIII. The pGL2 promoter vector was digested with the same restriction enzymes, and the SV40 promoter contained in this vector was released and removed. The PCR promoter fragments ligated into the vector, which was then transformed into competent DH5α bacteria (from Gibco/BRL). DNA for the promoter activity analyses, which are described below, was isolated from transformed bacterial clones using the Qiagen plasmid kit.

Example 10

The activity of the hTC promoter was analysed in transient transfections in eukaryotic cells.

All the work with eukaryotic cells was carried out at a sterile workstation. CHO-K1 and HEK 293 cells were obtained from the American Type Culture collection.

CHO-K1 cells were kept in DMEM Nut Mix F-12 cell culture medium (from Gibco-BRL, order number: 21331-020) containing 0.15% streptomycin/penicillin, 2 mM glutamine and 10% FCS (from Gibco-BRL).

HEK 293 cells were cultured in DMOD cell culture medium (from Gibco-BRL, order number: 41965-039) containing 0.15% streptomycin/penicillin, 2 mM glutamine and 10% FCS (from Gibco-BRL).

CHO-K1 and HEK 293 cells were cultured at 37°C in a water-saturated atmosphere while being gassed with 5% CO_2. When the cell lawn was confluent, the medium was sucked off, after which the cells were washed with PBS (100 mM KH_2PO_4 pH

7.2; 150 mM NaCl) and released by adding a trypsin-EDTA solution (from Gibco-BRL). The trypsin was inactivated by adding medium and the cell count was determined using a Neubauer counting chamber in order to plate out the cells at the desired density.

For the transfection, in each case 2x 10^5 HEK 293 cells were plated out, per well, in a 24-well cell culture plate. The HEK 293 medium was removed after 3 hours. For the transfection, up to 2.5 µg of plasmid DNA, 1 µg of a CMV ß-Gal plasmid construct (from Stratagene, order numner: 200388), 200 µl of serum-free medium and 10 µl of transfection reagent (DOTAP from Boehringer Mannheim) were incubated at room temperature for 15 minutes and then dropped uniformly onto the HEK 293 cells. 1.5 ml of medium were added after 3 hours. The medium was changed after 20 hours. After a further 24 hours, the cells were harvested for determining the luciferase activity and the ß-Gal activity. For this, the cells were lysed, at room temperature for 15 minutes, in the cell culture lysis reagent (25 mM Tris [pH 7.8] containing H_3PO_4; 2 mM CDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100). Twenty µl of this cell lysate were mixed with 100 µl of luciferase assay buffer (20 mM Tricin; 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$; 2.67 mM $MgSO_4$; 0.1 mM EDTA; 33.3 mM DTT; 270 µM coenzyme A; 470 µM luciferin, 530 µM ATP), and the light generated by the luciferase was measured.

In order to measure the ß-galactosidase activity, equal quantities of cell lysate and ß-galactosidase assay buffer (100 mM sodium phosphate buffer, pH 7.3; 1 mM $MgCl_2$; 50 mM ß-mercaptoethanol; 0.665 mg of ONPG/ml) were incubated at 37°C for at least 30 minutes or until a slight yellow coloration appeared. The reaction was stopped by adding 100 µl of 1 M Na_2CO_3, and the absorption was determined at 420 nm.

In order to analyse the hTC promoter, four hTC promoter sequence segments of differing length were cloned 5′ in front of the luciferase reporter gene (cf. Example 9).

The relative luciferase activities of two independent transfections in HEK 293 cells, using the constructs NPK8, NPK15, NPK22 and NPK27, are plotted in Fig. 11. Each experiment was carried out in duplicate. The standard deviation has also been given. The construct NPK 27 exhibits a luciferase activity which is 40 times higher than the basal activity of the promoterless luciferase control construct (pGL2-basic) and from 2 to 3 times higher than that of the SV40 promoter control construct (pGL2PRO). Interestingly, a luciferase activity which was from 2 to 3 times lower than that obtained with the NPK 27 construct was observed in cells which were transfected with longer hTC promoter constructs (NPK8, NPK15, NPK22). Similar results were also observed in CHO cells (data not shown).

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